MAXIMAL STEROIDOGENIC CAPACITY OF MOUSE LEYDIG CELLS

Kinetic analysis and dependence on protein kinase activation and cAMP accumulation

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Received 13 September 1979

1. Introduction

We have described a non-enzymic procedure for the isolation of pure mouse Leydig cells with retention of morphological and biochemical integrity [1]. The hCG-stimulated cells showed a >20-fold higher testosterone production than collagenase-dispersed rat Leydig cells (cf. [2,3]). In the absence of any phosphodiesterase inhibitors, they responded to nM doses of hCG with a >300-fold increase in intracellular cAMP. Since other systems react to the trophic hormone with a far less-pronounced increase in cAMP and still showed practically complete activation of protein kinase (cf. [4,5]), it was of interest to analyze the requirements of these Leydig cells for a maximal testosterone formation with respect to intracellular cAMP, bound cAMP (~R·cAMP) and protein kinase activation. This paper describes the transmission of the hormone message through the cAMP/protein kinase system by a kinetic analysis at different hCG concentrations and correlates the maximal rate of steroidogenesis with the time-dependent accumulation of intracellular cAMP, bound cAMP, and the activation of protein kinase. The data show that mouse Leydig cells require <33% of their total cAMP-dependent protein kinase activity, and <5% of the potential for the accumulation of intracellular cAMP to synthesize testerosterone at a maximal rate for many hours. In addition it is shown that excessive cAMP accumulation is accompanied by a progressive and parallel loss of (total) protein kinase and of receptor-bound cAMP from the cytosol which cannot be explained by a reassociation of the subunits to the inactive holoenzyme or a release to the medium.

2. Materials and methods

Highly purified hCG (13.791 IU/mg, 2nd Int. Standard hCG) was obtained from Serono, Rom, [^{33}P]phosphoric acid and ^{125}I-labeled cAMP (adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl 3-[^{125}I]iodotyrosine methyl ester) from New England Nuclear (Dreieichenhain), and Percoll from Pharmacia (Frankfurt).

2.1. *Preparation of Leydig cells*

Disaggregation of mouse testis cells was performed by a modification of the non-enzymic procedure first described in [6]: testes of adult mice (NMRI, 3–4 months old) were decapsulated without damaging the bulk of seminiferous tubules. The decapsulated testes were placed in Hepes-buffered minimum essential medium (Eagle, MEM), and carefully drawn 15–20-times into a 70 ml syringe provided with a silicone tubing ~6 mm diam. This process was repeated twice with reduced openings of the tubing (4 mm, 2 mm diam). During this treatment (22°C) the whole testis was dispersed into seminiferous tubules and testicular cells. The filtered suspension contained 20–40% Leydig cells, which were sedimented, resuspended in MEM and purified in Percoll density gradients as in [1]. All glass tubing was freshly siliconized. The yield of Leydig cells was usually 6–9 × 10^5 cells from one testis.

2.2. *Incubation of cells*

Cells (1.5 × 10^5) were routinely incubated in 400 μl MEM ± hCG at 36°C in a shaking water bath for an appropriate time. Subsequently they were sedi-

Elsevier/North-Holland Biomedical Press

mented (750 X *g*, 5 min), the supernatant was used for testosterone determination [7] and the pellet extracted either with 0.5 M perchloric acid for quantitation of intracellular cAMP, or stored at −80°C for the assay of protein kinase activity and protein-bound cAMP.

2.3. *Determination of cAMP*

cAMP was analyzed by the method in [8] as modified [9]. Cell extract, 200 μl in 0.5 M $HClO_4$ (see above) was neutralized with 100 μl 0.71 M K_3PO_4 and the precipitated $KClO_4$ was removed by centrifugation. Supernatant (100 μl) was vigorously mixed with 5 μl freshly prepared acetylating reagent consisting of 1 part acetic acid anhydride and 2.7 parts triethylamine. cAMP standards were treated the same way.

2.4. *Preparation of charcoal supernatants*

To the frozen cell pellet (1.5 X 10^5 cells) 300 μl ice-cold charcoal suspension (1% in 10 mM acetate buffer (pH 5.8) containing 0.1% bovine serum albumin and 70 mM NaCl) was added and the mixture was sonicated for 10 s at 0°C in a beaker resonator (Cup-Horn, Branson Sonifier). After standing for 6 min in ice, the mixture was centrifuged (14 000 X *g*, 5 min, 4°C). One aliquot of the clear charcoal supernatant was extracted with $HClO_4$ and analyzed for cAMP (see above), in another aliquot protein kinase activity was determined without delay.

2.5. *Protein kinase activity*

Enzyme activity was routinely assayed for 5 min at 30°C. The reaction was started by the addition of 20 μl charcoal supernatant (≈ 10^4 cells) to 100 μl incubation mixture containing 20 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 0.5 mM MIX, 0.3 mM EGTA, 50 mM [^{33}P]ATP (2 X 10^6 cpm), 1.0 mg histone/ml and where indicated 2 μM cAMP. Incubation was stopped by spotting 100 μl mixture on paper discs (2 X 3 cm, Whatman ET 31) pre-soaked with 5 mM ATP and dried. This procedure led to a high reproducibility and low blanks. Washing in trichloroacetic acid solutions was performed according to [10]. The dried paper discs were counted in 15 ml dioxane based scintillation cocktail. [γ-^{33}P]ATP was prepared by the procedure in [11] as modified [12].

3. Results

3.1. *In pure Leydig cells, hCG-induced testosterone formation is associated with elevation of intracellular cAMP levels*

When pure mouse Leydig cells prepared by the non-enzymic procedure [1] were stimulated by increasing concentrations of hCG, they responded with an increasing accumulation of intracellular cAMP, and increasing testosterone formation. Although half-maximal steroidogenesis occurred at ~7-times lower hormone concentrations than required for half-maximal cAMP formation (fig.1, inset), there was a significant increase in cAMP associated even with the lowest increase in testosterone synthesis (fig.1). This dependency obtained at 3 h after the addition of the trophic hormone indicated that either a relatively small rise of intracellular cAMP was sufficient to elicit maximal steroidogenesis or a high but transient increase in cAMP served as a signal for a continued steroid synthesis at an elevated rate. Therefore, a kinetic analysis of the action of different doses of the trophic hormone was performed.

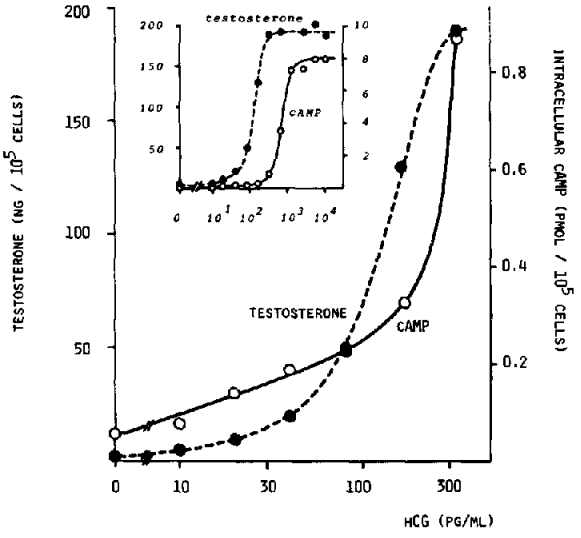

Fig.1. Accumulation of intracellular cAMP and testosterone synthesis in pure mouse Leydig cells in response to different hCG concentrations. Cells (10^5) were incubated for 3 h at 36°C under the conditions in section 2. Inset: Comprehensive data of the same experiment. Mean values from triplicates.

3.2. *Kinetic analysis of cAMP formation, protein kinase activation and testosterone synthesis*

Leydig cells stimulated with a high dose of hCG (50 ng/ml ≈1000 pM) responded with an immediate and excessive accumulation of intracellular cAMP reaching peak values 300-times above control levels within 15 min (fig.2A). This rapid formation of cAMP was followed by a decline to a nearly constant level of ~20% of the maximal value. The same hormone concentration also provoked a prompt and nearly complete activation of protein kinase (fig.2B). The small decrease in protein kinase activity ratio seen at later periods may not be real since under these conditions, a loss in total C activity was observed on a background of some cAMP-independent protein kinase activity (see below).

Testosterone synthesis was significantly increased by 1000 pM hCG already after 5 min. It reached a maximal rate after ~30 min which remained constant for ⩾5 h (fig.2C). The maximal rate of testosterone production of purified mouse Leydig cells obtained from 4 kinetic experiments was 605 ± 46 ng . h^{-1} . 10^6 $cells^{-1}$. This is >20-times higher than values obtained with collagenase-dispersed rat Leydig cells (cf. [2,3]).

Stimulation of the cells with 100-times lower hCG concentration (0.5 ng/ml ≈ 10 pM) resulted in a much slower increase in intracellular cAMP. Maximal levels (20-times control values) were only seen after 1–2 h incubation. Longer incubation periods again led to a slow decrease of intracellular cAMP. Under these conditions only a partial (33.3% of total capacity) activation of protein kinase was reached which remained constant between 0.75 and 4 h incubation (fig.2B). This rather small degree of protein kinase activation, however, was sufficient to guarantee a maximal and unretarded testosterone synthesis (fig.2C). Only at very low concentrations of the trophic hormone (1 pM hCG) a marked retardation of cAMP formation was seen, significant elevation of total intracellular cAMP being visible after 25 min (fig.2A, inset). The maximum, (<2-times the control values) appeared only after 2–3 h and remained nearly constant during the rest of the incubation period. The low hormone concentration also caused a rather small and somewhat retarded activation of protein kinase, and a retarded testosterone production. Like in the time course of cAMP accumulation a significant increase in testosterone synthesis did not

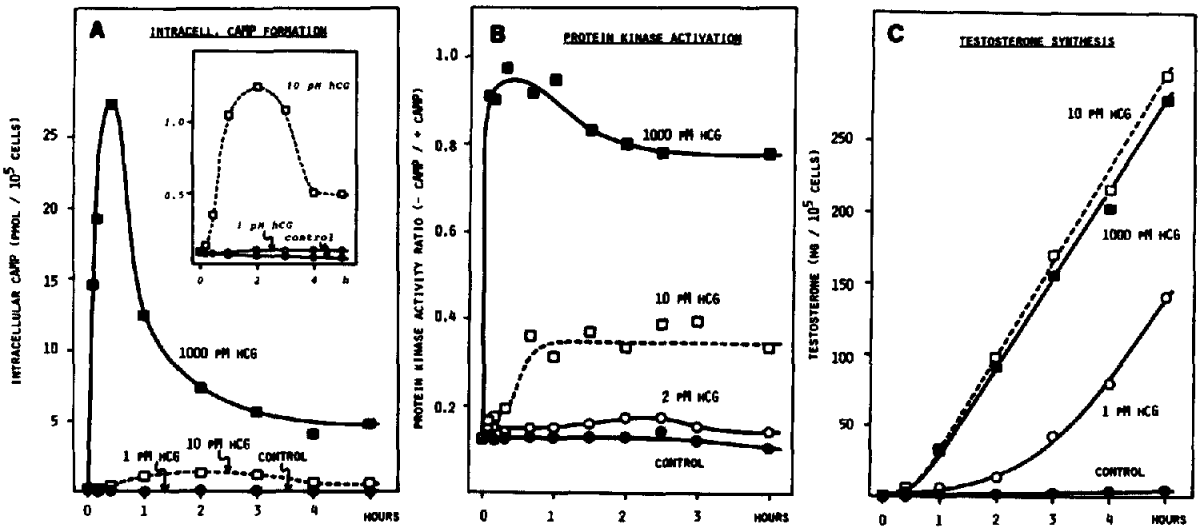

Fig.2. Kinetic analysis of hCG-induced increase in intracellular cAMP accumulation (A), protein kinase activation (B) and testosterone production (C). Cells (1.5 × 10^5) were incubated with 3 different hCG concentrations for various times as indicated. Cells were homogenized in the presence of charcoal and salt, and enzyme activity was assayed in the supernatant with and without 2 μM cAMP (see section 2). Protein kinase activation is expressed as the activity ratio (activity in the absence to that in the presence of 2 μM cAMP). Inset: Data of fig.2A at larger scale.

occur before 25 min reaching about half-maximal values after 5 h incubation. This and other testosterone kinetics performed with submaximal hCG concentrations indicated that the reduced testosterone production determined after several hours of incubation was more a result of a delayed onset than of a reduced rate of steroidogenesis.

3.3. *Receptor-bound cAMP and absolute protein kinase activity during hCG stimulation*

According to the equation*:

$$R_2 \cdot C_2 + 4\ cAMP \rightleftharpoons R_2 \cdot cAMP_4 + 2\ C$$

the biologically active form of intracellular cAMP is the fraction which is bound with high affinity to the regulatory subunits of the protein kinases. It was therefore of interest to include this fraction of 'bound cAMP' which in other systems proved to be directly correlated to protein kinase activation [4,13]. Furthermore, the activity ratio of protein kinase as measured in the presence and absence of added cAMP may obscure actual changes in absolute protein kinase activity in extracts due to translocation or other phenomena (cf. [14,15]). Protein-bound cAMP (~R·cAMP) and protein kinase activity were therefore determined over 4 h after the addition of different doses of the trophic hormone. In order to obtain comparable results both parameters were assayed in aliquots of the same charcoal supernatant. These conditions also excluded artificial activation of protein kinase by high concentrations of cAMP released from stimulated cells into the homogenization medium during cell disruption. As shown in fig.3, stimulation of mouse Leydig cells with different hCG concentrations resulted in very similar kinetics for bound cAMP and protein kinase activity. These kinetics, however, were dissimilar to the kinetics of the protein kinase activity ratio (−/+ cAMP, fig.2B). While the highest hCG dose lead to a maximal activity ratio in <2 min, and maintained a high ratio throughout the incubation period, the absolute values of free C activity (fig.3) were also maximal in <2 min, but declined there after continuously throughout the incubation period. This decrease was concentration-dependent being seen only at hCG doses leading to excessive accumulation of cAMP. Even 2 min after hCG addition, a significant decrease in protein kinase activity compared to the total activity of unstimulated cells was noticed.

* Recently, the stoichiometry of cAMP binding to the regulatory subunits for R II [16,17] and R I [18] has been re-evaluated and found to correspond to 2 binding sites/R monomer

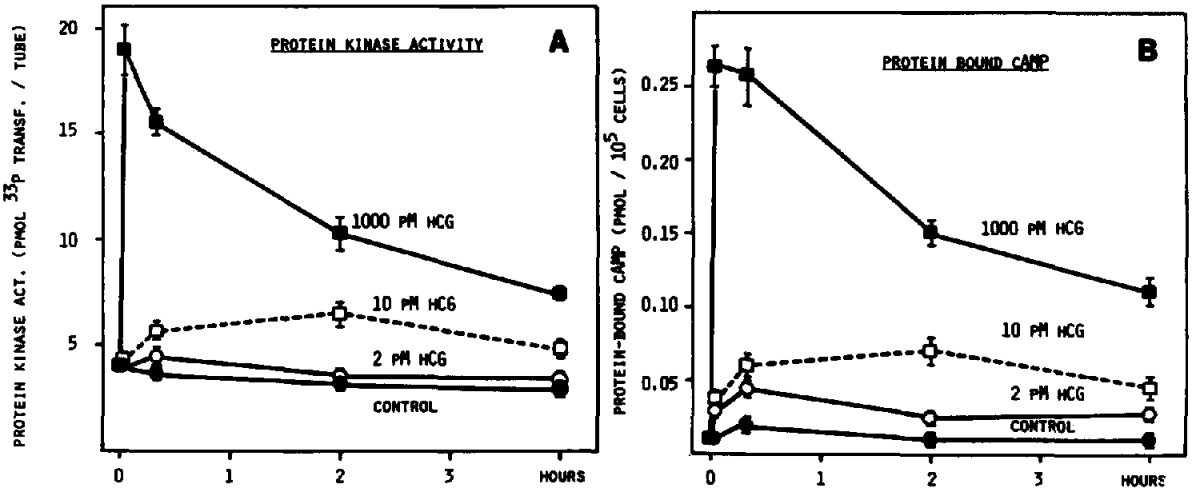

Fig.3. Time course of hCG action on absolute protein kinase activity (A), and on protein-bound cAMP (B). Sedimented cells were homogenized in the presence of charcoal and NaCl and both parameters analyzed in the same supernatant as in section 2. Mean values ± SEM of quadruplicates.

The loss of absolute enzyme activity and of bound cAMP was not due to a reassociation of protein kinase subunits to the inactive holoenzyme. Determination of the samples in the presence of added cAMP showed that total kinase activity, too, had progressively disappeared from the supernatant. There was also no leakage of protein kinase to the extracellular space as determined by direct analysis of the incubation media. This was also true for the receptor-bound cAMP which proved again to be a direct measure of the amount of active C subunit being linearly correlated with this parameter under all experimental conditions (fig.4). Progressive leakage of the cells during incubation is therefore an unlikely explanation for the disappearance of C and R·cAMP at high cAMP concentrations.

The kinetic analyses of hCG-induced, protein kinase-mediated steroidogenesis in these highly responsive cells clearly show that the cellular capacities are far in excess of what is needed for a long-standing maximal rate of testosterone synthesis. Whether the time-dependent loss of C and R·cAMP from the soluble fraction at high hCG concentrations (producing highly excessive intracellular cAMP levels) is a form of desensitization, or a translocation to the particle fraction, or connected to still other mechanisms remains to be determined.

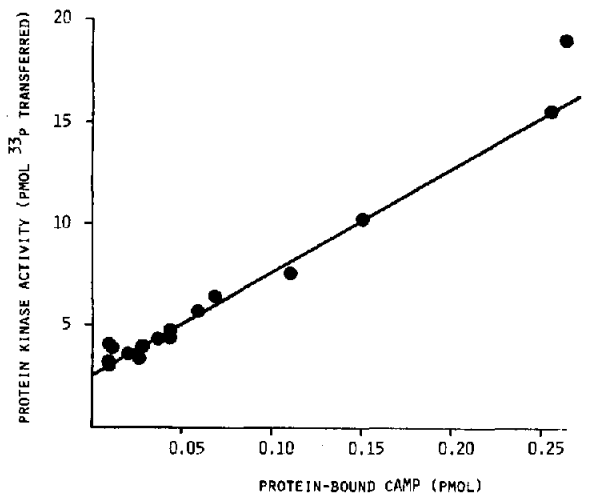

Fig.4. Correlation of protein kinase activity and protein bound cAMP. The values were derived from the experiment in fig.3.

Acknowledgements

We would like to thank Frau E. Spikofski for assistance in animal care and handling. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 34.

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